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REVIEW

Measurement and reporting of data in applied biocatalysis ☆

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Abstract
Enzymes are used in various sectors of industry. Invariably, industries procure these enzymes from the vendors. Such commercial preparations are seldom of high purity. Hence reporting catalytic data obtained with such enzyme preparations poses some special challenges in addition to the usual ones associated with enzymology. This chapter discusses some of these challenges.
The purity of an enzyme is the first such issue in view of the impure nature of the commercial preparations of the enzymes. One should be cautious in relying upon the kinetic data obtained with such preparations. Estimation of the protein content can be challenging as often the nature of the interfering substances, if any, is unknown. Measurement of the total number of activity units requires careful attention to many possible pitfalls. It is also necessary to be very clear about whether activity per unit mass relates to the weight of the solid or protein.
Initial rates do not always give the correct picture about the usefulness of an enzyme for a particular reaction. Hence, the complete progress curve should be reported. Similarly, the operational stability under actual conditions of the application is more useful than stability in aqueous buffers. High activity of an enzyme preparation in low-water systems may be due to the reduction of mass transfer constraints rather than the high stability of the enzyme in such systems. Finally, the phenomena of moonlighting proteins and catalytic promiscuity have thrown fresh challenges as these questions the fundamental paradigm of one protein-one function.

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Contents

Introduction

Biocatalysis is an important component of development of sustainable chemical processes (Schumacher et al., 2006; Sell and Ulber, 2006). Jaeger (2004), in the early days of white biotechnology, talked about enzyme catalyzed processes replacing "fire and sword" chemistry which relies upon harsh conditions. Only few decades back, Whitesides and Wong (1983) wrote an article about what enzymes can do and what they cannot do. Progress in biocatalysis almost makes one believe that there is no reaction for which an enzyme cannot be found or engineered. Recent reports show that the earlier notion that new enzyme activities are no longer evolving in nature may be wrong (Janssen et al., 2005). Techniques like directed evolution promise that given an application, an enzyme/biocatalyst can be designed (Arnold and Georgiou, 2003a, 2003b). Hence applied biocatalysis has definitely come of an age. Enzymes are used in various industrial sectors: food, textile, leather, biofuels, drugs and pharmaceuticals (Table 1). Also, these applications may involve the use of enzymes/biocatalyst in so called nonconventional media: organic media (Gupta, 1992; Vulfson et al., 2001) reverse micelles (Orlich and Schomäcker, 2002) and ionic liquids (Park and Kazlauskas, 2003; Shah and Gupta, 2007a).

Many enzyme preparations are commercially available in either free form or in immobilized form. These preparations are either sold in solid form or as solutions or suspensions. Often, for proprietary reasons, their constituents (other than the enzyme part) are not known to the user. Worse still, units are not properly defined or may differ from vendor to vendor or even from preparation to preparation offered by the same vendor. Hence, there is an urgent need for evolving norms for reporting data so that science can consist of reproducible data. This chapter attempts to identify some problems and challenges while describing quantitative results about a particular application of any enzyme. In many cases, "solutions" to the problems are easy provided all stake holders (scientists, enzyme vendors, industries and journals!) agree. In other cases, we need to search for the best possible solutions. Many issues discussed here are not restricted to industrial enzymology. However, industrial enzymology does involve some additional pitfalls.

Purity of the enzyme preparation

What is a pure enzyme? Curiously enough, while older text books of biochemistry (Mahler and Cordes, 1966) discussed this issue, the current text books of biochemistry pay scant attention to this question and treat this as more or less a non-issue. Perhaps, it could be the result of the paradigm shift in the way protein purification is carried out.

In early times, protein purification protocols invariably used to be multi-step processes. They followed a more or less set sequence of unit processes: precipitation → ion exchange chromatography → gel filtration → (another exchange chromatography) → affinity chromatography (Gupta, 2002). These multi-step protocols raised the cost of production of a protein to the point where the downstream component could

Table 1 Industrial enzymes (Walsh and Headon, 1994; Straathof and Adlercreutz, 2000). The following is an illustrative list of enzymes being used in the various industrial sectors and other applications.

Enzyme	Applications
Proteases	Detergents, brewing and baking, cheese production, leather processing, digestive aids, debriding, ester hydrolysis, peptide synthesis
Amylases	Starch degradation, bioethanol and other fermentation products, textile industries
Pectinases	Clarification of fruit juices, production of baby foods
Lactase	Production of low lactose milk, whey lactose hydrolysis followed by fermentation to provide alcohol
Glucose isomerase	Production of an industrial sweetner called High Fructose Corn Sirup
Penicillin acylase	Designing semi-synthetic penicillins
Lipases	Hydrolysis of fats/oils, production of designer fats/oils, biodiesel, synthesis and racemic resolution of drug intermediates and agrochemicals
Phytase	Animal feed additive

constitute >80% of the overall production costs (Przybycien et al., 2004). Many strategies have been developed over the years to reduce the cost of protein purification (Przybycien et al., 2004). These efforts have been multi-disciplinary in nature. Biochemical engineers and material scientists have contributed a lot to these developments.

The latter discipline, for example, is providing nanomaterials which can be used as support for separation of enzymes (Bucak et al., 2003; Ditsch et al., 2006). Some key trends have been:

- Integration of upstream and downstream components (Gupta and Mattiasson, 1994; Mondal et al., 2006).
- Developing techniques to deal with crude and dirty feed with particulate matter (Mattiasson, 1999; Mondal et al., 2003; Roy and Gupta, 2000; Sharma and Gupta, 2002; Teotia and Gupta, 2001).
- Bringing up affinity-based separation step much earlier in the purification process. This in turn has involved relying upon non-chromatographic methods (Przybycien et al., 2004).
- Lately, the most frequently used approach for purification of recombinant proteins is the use of fusion tags or affinity tags. That also is an affinity based approach and can be used in both chromatographic and non-chromatographic modes (Lichty et al., 2005).

As most of proteins or enzymes are produced by recombinant route, protein purification has increasingly come to be viewed, at least in the academic sector, simply as use of an affinity tag along with the corresponding affinity media. Furthermore, this is generally carried out by using a commercial kit. If one does not work, another one is tried! Simultaneously, the older view of using multiple criteria for establishing the purity of a protein has been replaced by being satisfied with a single band on SDS-PAGE. This often can lead to an unsatisfactory situation. The older approach of evaluating protein purity by PAGE carried out at at least two widely different pH values, and ultra centrifugal analysis was much more sound.

What is more, there are many ambiguities associated with the way SDS-PAGE is carried out and there does not seem to be an agreement (one is generally at the mercy of the wisdom of the peer review). How much "pure protein" should be loaded as compared to the crude protein preparation lane? Some people advocate equal amount of protein in both lanes. If the crude has 10% of the desired protein; the "pure protein" lane ends up having a 10-fold more intense band. Some people during peer review have a problem with that especially since more often than not the "pure protein" in such cases would show a rather broad band. What may be desirable is to load two or more widely different concentrations of proteins 0.5 ×, 1 ×, 2 × (depending upon how crude the starting material was). One of the bands of the pure protein should be sharp and intense; another should be an "overload" to ensure that all significant traces of impurities can be detected. Coomassie Blue stain seems to be widely accepted "gold standard". However, a protein preparation found to show a single band on SDS-PAGE by Coomassie Blue may actually show several bands upon use of silver stain (Walker, 2002). This is widely known. Well established journals seem to accept structural work if the SDS-PAGE (with Coomassie Blue stain) show >95% purity.

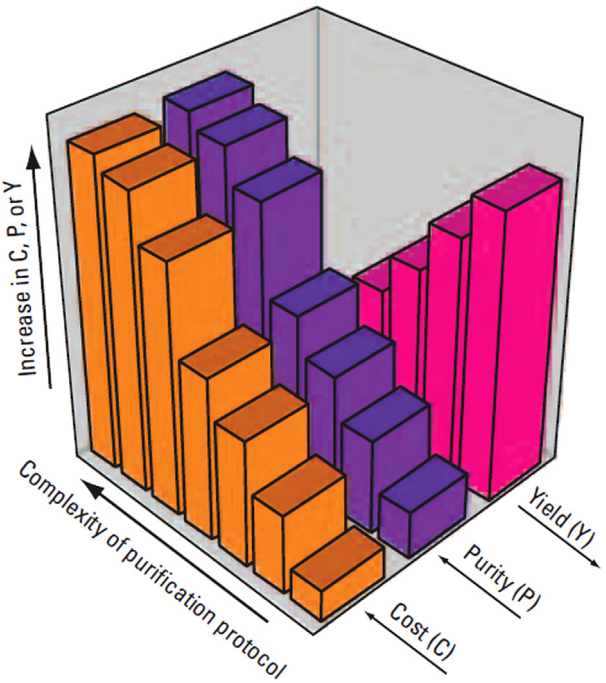

Figure 1 With an increase in the complexity of the purification protocol, there is an increase in the purity of the protein and the cost of the purification protocol. This also results in the decrease in the yield of the desired protein.
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There is another disturbing practice which is occasionally seen that the band of the protein is shown at far end of the lane. This rules out detecting the presence of any proteolytic fragments or contaminating proteins of lower molecular weight.

Not all applications of the proteins require the same level of purity. This is an important point since there is a three way trade-off between purity vs. number of steps vs. cost of production (Figure 1).

Industrial enzymes used in many industries do not require high purity. Reasonable level of specific activity is sufficient. Proteins used for pharmaceutical applications (e.g. monoclonal antibodies or clot busters, hormones, etc.) not only require extremely high purity; regulatory agencies require that these preparations are specifically free of certain contaminants (Anicetti and Hancock, 1994; Walsh and Headon, 1994) (Table 2).

There is also a fairly widespread practice of measuring K_m, V_{max} and stability of proteins which are fairly impure. Unless, the preparation is standardized with respect to contaminants (like in commercially available industrial enzymes), such data actually cannot be relied upon (the reason for this is explained later on).

Finally, as may be clear from the above discussion, protein purity is a relative term. One of the most well characterized enzymes is bovine pancreatic RNase A (Richards and Wyckoff, 1971). Most of the work, including X-ray crystallography, has been carried out with a "pure"

Table 2 Impurities in pharmaceutical proteins (from Anicetti and Hancock, 1994; Walsh and Headon, 1994). Pharmaceutical proteins include blood products, hormonal preparations, cytokines, enzymes, vaccines and antibodies including monoclonal antibodies. The following impurities have to be removed or reduced to a level prescribed by the regulatory agencies.

Impurity	Comments
Endotoxins	These are pyrogenic substances and their presence can be detected by Rabbit Pyrogen Test or Limulus Amebocyte Lysate (LAL) test.
Infectious agents such as microorganisms and viral particles	Their presence can lead to severe infection. These contaminants can be tested by reverse transcriptase assay. Cell culture cytopathic effects and electron microscopy.
Foreign proteins	These include host cell proteins, proteins present in the media and antibodies. Immunoassays or electrophoretic analysis can be used to detect these.
DNA (less than 10 pg per therapeutic dose are in general considered acceptable)	Can cause immune response. Hybridization assays can detect it. Numerous other methods available.
Product variants (these include products arising from protein instability during the production)	These include isoproteins, oxidation products, deamidation products, aggregates or proteolytic fragments. Various methods of protein detection can be used.

preparation obtained by a final ion-exchange chromatographic step (Richards and Wyckoff, 1971). However, this preparation shows multiple proteins when subjected to multiple counter-current distribution process (Richards and Wyckoff, 1971)!

In general, crystallization can be both a purification strategy (Przybycien et al., 2004) as well as a criterion of reasonable purity (Dixon et al., 1979). Precipitation, both with and without an interface with affinity interactions is another efficient, simple and scalable approach (Mondal et al., 2006; Mondal and Gupta, 2006; Niederauer and Glatz, 1992). Most of the industrial enzymes these days are produced by recombinant methods wherein overexpression leads to a considerably less heterogeneous protein preparation. Many proteins upon overexpression in *Escherichia coli* as host end up as inclusion bodies. In recent years, in many cases these inclusion bodies are being considered as carrier-free immobilized preparation of fairly pure enzymes (Garcia-Fruitos et al., 2012).

Protein estimation

One of the key parameters in biocatalysis is the amount of protein present in the biocatalyst preparation. There are various good reviews and articles in protocol books (Walker, 2002) which give details of various protein estimation methods. Many scientists, these days also rely upon a gel scanner to estimate protein in a given sample by running a SDS-PAGE. The few features of these methods are sometimes less clearly taken into account than desirable.

1. Most of the protein estimation methods rely upon the color-generating response of the protein during a chemical reaction (e.g. Biuret, Lowry or BCA methods) (Walker, 2002) or physical interaction with a compound (e.g. frequently used dye-binding assay) (Bradford, 1976). Different proteins respond in a quantitatively different way. In this respect, Biuret is an exception as it gives relatively uniform response for most of proteins. This is much less sensitive than other methods (Scopes, 1994). However, most of the industrial enzymes contain a good amount of protein/g, so Biuret actually may be a good option. Most of the other methods give the relative protein concentration. For example, it is a general practice to say that a particular protein estimation method was employed and BSA was used for a standard curve. The color-generating response by the protein can be very significantly different from BSA. This is not a cause of worry as we mostly track *change* in protein concentration during any operation/process. For example, during protein purification, we are only concerned with fold purification starting with a crude preparation. So, the relative protein concentration value should be good enough. However, when we calculate the amount of protein expressed and obtained as inclusion bodies (Garcia-Fruitos et al., 2012), we tend to overlook that we are not talking of absolute protein concentration.
2. With most of the protein estimation methods, a list of common interfering substances is known (Walker, 2002) and one can run a control while dealing with any known interfering substances. The problem is that a crude homogenate or a feed from a fermenter would contain many unknown substances. It is a good practice to dialyze extensively to get rid of at least low molecular-weight substances before carrying out the protein estimation. Even after this any large molecular weight substances remaining in the crude fraction are going to affect the protein estimation. Protein extracts from the plant sources are often rich is plant phenolics and tannins. While many suggestions have been made to minimize oxidation of these compounds (Pierpoint, 1996), it is often not possible to suppress those oxidative reactions completely. As a result, not only can quinoid products modify the proteins (Gupta and Vithayathil, 1980; Vithayathil and Gupta, 1981), but these compounds themselves oligomerize to produce melanins or their precursors (Pierpoint, 1996). There are no easy solutions; the best one can do is to be aware of these "artifacts" in the data being reported.
3. A corollary of what is said above is that different protein estimation methods are likely to give different values.

This would arise from the fact that color-generating response of a protein may differ from BSA to different extents in different protein estimation methods. Hence, quantitative estimation of absolute concentration of protein can be tricky. The same protein (if available) as being estimated should be used as a standard.

Activity units

The amounts of an enzyme present in a given sample, reaction system or bioreactor is obviously an important parameter. If the reaction condition obeys Michaelis–Menten kinetics, it is implied that [E]«[S]. Ideally, if the amount of enzyme is increased *x* times, the initial rate is expected to increase *x* times. In reality, it may not happen. The plot of velocity vs. [*E*] curve may have an increasing slope (display a lag period or a slow phase) if:

(a) The oligomeric form of an enzyme has higher activity or if the subunits of the enzyme dissociate in dilute solutions.
(b) The enzyme molecules bind to the surface of the container due to various nonspecific interactions. A well-known illustration of this phenomenon is observed during ELISA wherein micro-plates normally are pre-coated by BSA etc. to prevent nonspecific adsorption of antigen, antibody or ELISA reagent (Benjamini et al., 1996). Another manifestation of this phenomenon is chromatography on dye-affinity columns. Shielding chromatography i.e. pre-coating the matrix with some polymer before initiating protein binding has been described to address this concern (Galaev and Mattiasson, 1994). So, non-specific binding by enzymes to various surfaces is more prevalent than realized. Indeed the widespread use of simple adsorption to immobilize enzymes to a wide range of support materials is evidence of this.
(c) The enzyme preparation may be contaminated with an activator. This activator could even be a simple metal ion which is inadvertently present in the buffer. So, as the activator concentration reaches a threshold, the reaction velocity becomes much faster.

On the other hand, the velocity vs. [*E*] curve may have a decreasing slope (i.e. the velocity slows down with increase in [*E*]) because:

(i) The enzyme has a tendency to aggregate. These aggregates may be soluble. So, no visible precipitation is observed. Earlier, it was believed that extensive aggregation requires unfolding of the protein chain. Now, there is growing evidence that even "native-like structures" may aggregate (Bemporad et al., 2012). Intrinsically disordered proteins (IDP), of course, constitute an extreme case in this regard (Uversky, 2011). Aggregates are generally inactive although recently alpha chymotrypsin subjected to three-phase partitioning (TPP) (Rather et al., 2012) has been shown to form aggregates which are more active then monomeric alpha chymotrypsin (Rather et al., 2012). α-Chymotrypsin is also a good example which shows high propensity for forming soluble aggregates even in simple buffers (Ghaouar et al., 2010; Rezaei-Ghaleh et al., 2008).
(ii) The enzyme preparation may contain an inhibitor as a contaminant. The proportion of this contaminant, nature of its inhibition and its inhibition constant K_i may be such that the inhibition is rendered less effective at higher [*E*].
(iii) In a coupled enzyme assay, the second auxiliary enzyme may not be sufficient. Measuring glucose oxidase activity by peroxidase coupled assay (Bisswanger, 2011) is a well-known system.

For a more detailed discussion on this, excellent references are Eisenthal and Danson (2002), Purich (2010) and Tipton (1985).

In any case, the amount of the enzyme can be expressed as total units of activity or % weight of the preparation. In traditional enzymology, commonly practised in the academic sector, the former parameter is generally used to track the loss or retention of enzyme amount at each step of purification. Earlier, an enzyme purification table used to be mandatory while reporting purification of an enzyme. Sadly, it is frequently missing in recent publications. Not providing an enzyme purification table obscures the issue of how good a purification protocol is. Several formats of enzyme purification tables are still described in some good books (Scopes, 1994), the one most recommended is as originally given in the iconic book by Dixon et al. (1979).

While units are expected to be international units (Bains, 2002), quite often the term enzyme unit is used in an arbitrary fashion. It is preferable to use I.U. or katals (see also Cornish-Bowden's contribution on Analysis and Interpretation of Enzyme Kinetic Data, 2014 and Tipton et al., 2014). If not, the unit used must be comprehensively defined (see below in this chapter for a discussion on moonlighting protein and promiscuity, situations where there are difficulties in using I.U.).

Sometimes, an enzyme preparation is expressed in terms of its specific activity. The specific activity is defined as units/mg protein. This term allows one to track purity of a protein during a protein purification protocol. Obviously, higher the specific activity at any step, greater is the purity.

In industrial enzymology, the parameter specific activity creates confusion. The commercially available enzymes, even in the free-state, are invariably mixed with many foreign substances. The composition of the preparation is often proprietary information. Quite often, a stabilizer of unspecified nature is present. These substances (additives) may interfere with most of the protein estimation methods. The same issue of course arises in protein purification work which almost always starts with fairly crude mixture ("crude extract").

As the nature and extent of interference cannot be established, one cannot run controls to take care of the positive or negative contribution of the additives to the value obtained during the activity estimation method.

Quite often, the commercial preparation is an immobilized one. The amount of protein immobilized per gram of solid support matrix is seldom specified. This has relevance in interpreting any reported data. Quite often, different enzyme preparations are "screened" for finding out the best enzyme preparation for a given biotransformation (Furutani et al., 1995; Kapoor et al., 2012). For example, screening of commercial preparations of lipases for obtaining the best conversion of oil to biodiesel is quite common (Nelson et al., 1996; Shah et al., 2004; Shah and Gupta, 2007b). Similarly, lipases from different sources are increasingly screened for obtaining the best yield in a promiscuous reaction (Lai et al., 2010; Li et al., 2008). Invariably, initial rates are compared before the choice for the best biocatalyst is made. This may not necessarily be the best choice as initial rates are just that: rates yet to be affected by multiple factors which start operating as the reaction progresses (see later for a discussion on importance of complete progress curve). However, comparing either initial rates (which has "per mg" of the biocatalyst as a part of its units) or even the conversions and yields from two different commercial preparations is actually comparing apples and pears! Foresti and Ferreira (2005a) have discussed this issue in the context of lipase-catalyzed reactions. However, the points raised have wider implications particularly in the context of industrial enzymology.

- Strictly speaking, the terms "enzyme", "lipase" and "protein" are not interchangeable. The first one is the total weight of the preparation; "protein" is the total amount of protein in the preparation on a weight basis, this can constitute a very small percentage of the total weight of the "enzyme". Lipase, of course, refers to the amount of pure lipase present in the "enzyme". This often is an unknown quantity in a commercial enzyme preparation. It is, however, a common practice to use the term lipase for the total amount of protein, that is, the amount of impure lipase. Often, one has to rely upon the context to understand what may be meant.
- While describing (preparation of) an immobilized enzyme, different ways of reporting of the enzyme load are possible: gram of lipase per gram of support, gram of lipase per gram of the biocatalyst preparation, units of enzyme activity per gram of support or unit of enzyme activity per gram of the biocatalyst preparation. The last two, of course, may not differ much in values as a biocatalyst preparation consists of generally $>90\%$ support.
- Quite often, the support is poorly described. Celite is often used as the support for biocatalyst preparations (Adlercreutz, 1991; Vulfson et al., 2001). However, there are many different materials that are given the name celite, with various qualifiers and codes, and the nature of the immobilized preparation could very much depend upon the type of celite.
- Protein bound to the matrix is often calculated by subtracting the protein remaining in the supernatant from the initial amount of protein taken. One immediate issue arises here when little of the supplied protein is immobilized. The calculation now involves subtraction of two rather similar values, the error in the difference will, by usual statistical formulae, be very much greater than that in the measured concentrations, and is often large enough to make the result almost meaningless. Furthermore, the supernatant may contain substances which may interfere in the protein estimation method. For example, glutaraldehyde is one of the most frequently used cross-linking agents during immobilization procedures (Cao et al., 2000; Guisan, 2006). The glutaraldehyde remaining in the solution would interfere with many protein estimation methods. More likely to be missed is the fact that protein in the supernatant may have undergone various kinds of changes which would significantly affect its extinction coefficient in the ultraviolet spectroscopy or its color response during various protein estimation methods.
- Hydrolysis of tributyrin is an accepted assay for lipase activity. However, all lipases may not show adequate activity towards this triglyceride of the short chain alcohol (Kapoor and Gupta, 2012). Foresti and Ferreira (2005a,b) point out that *Candida antarctica* lipase B (a very frequently used form of lipase from Novozyme) shows poor activity for assaying hydrolysis in general. So, perhaps one needs to adopt a synthetic reaction (in low-water media) for this enzyme (Foresti and Ferreira, 2005b). Again, its specificity towards alcohols should be taken into account before adopting an assay for universal use.
- The conversion data is given in the literature in various ways: mole h^{-1}, mole h^{-1} g catalyst^{-1}, % conversion, and yield. The adequate information to compare these various values is seldom given. Sometimes, even time and amount of catalyst used are not explicitly stated. The use of RSM (Response Surface Methodology) to optimize the conversion has become quite popular. Often, one has to look very closely, to figure out what was the optimum conditions ultimately arrived at! This is particularly common in publications in which results show that RSM did not really help much.
- How does one compare the % conversions obtained in a solvent-free system with corresponding values in which solvent has been used? The standard practice is to keep the moles of reactant(s) the same and add the solvent. That changes molar concentrations of the reactants and obviously would affect the outcome.
- The rate of the uncatalysed reaction is rarely reported. It is because it is universally understood that enzymes accelerate the reactions tremendously. Foresti and Ferreira (2005a,b) point out that the concentrations of the substrates in the solvent-free systems are very high and the uncatalysed reaction may become significant. The reactions involving catalytic promiscuity especially require one to be extremely careful on this issue. It is necessary to show that the inactivated enzyme did not catalyze the reaction. In some reactions involving catalytic promiscuity, many additives (for example, bases) have been shown to further accelerate the enzyme-catalyzed reaction (Kapoor and Gupta, 2012). It is absolutely essential that two controls are carried out: just the additive (without any enzyme) and additive plus inactivated enzyme catalyzed reaction.
- In equilibrium-controlled reactions, the final % conversion which does not increase further with time is generally used to estimate the equilibrium conversion. This

overlooks the issue of operational stability of the enzyme. The accepted "gold standard" method is to show that the same equilibrium mixture is reached by starting the reaction from opposite directions.

- Recently, a decarboxylative aldol condensation was reported as a promiscuous reaction (Feng et al., 2009). A leading worker contradicted this report (Evitt and Bornscheuer, 2011) and argued that no promiscuity was involved. It was a normal catalysis followed by an uncatalysed reaction. The whole issue hinged on the presence or absence of water during the reaction. The reaction was found to occur under anhydrous condition as well; again raising the possibility that it was an enzyme-catalyzed promiscuous reaction (Kapoor et al., 2012). This is a good example of inherent challenges involved in reporting data on enzyme-catalyzed reactions in non-conventional media.

Foresti and Ferreira (2005a,b) have outlined how to avoid some of the above pitfalls by careful considerations while designing the experiments.

Importance of the complete progress curve

The efficiency of the enzymes is generally expressed in terms of initial rates. This is more or less the norm when the workers describe a more efficient biocatalyst design or formulation for catalysis in low-water media (Straathof and Adlercreutz, 2000; Vulfson et al., 2001). Engineered enzymes by site-directed mutagenesis or directed evolution are also generally evaluated in terms of initial rates. The initial rate, by definition, is the early initial and linear portion of product concentration vs. time graph.

In aqueous buffers, this linearity usually persists to at least till 5% conversion has taken place (Purich, 2010). In low-water media, conversions are much slower and one can observe linearity up to 20–30% of the conversion (Solanki and Gupta, 2008, 2011). Reactions which display a lag phase or a burst phase pose problems in accurate estimation of the initial rates. In many cases, the observed lag phase kinetic behavior could be an artifact and could arise from poor mixing and/or poor maintenance of the reaction temperature. In industrial enzymology, sometimes one has to deal with multi-substrate enzyme-catalyzed reactions. In such cases, the initial rate measurements depend upon whether the random or ordered mechanisms are involved. An excellent and comprehensive treatment for various possibilities is available at many places (Dixon et al., 1979; Eisenthal and Danson, 2002; Purich, 2010).

While the initial rate is a useful parameter for practical applications, a complete progress curve of the bioconversion or biotransformation is desirable, particularly in industrial enzymology. To be practically useful, a high conversion is desirable, often greater than 90%. An enzyme and reaction mixture that proceeds rapidly to 5% conversion, but then slows dramatically, will be less favoured than one that proceeds more slowly initially, but remains close to linear to high conversion. The velocity of the reaction falls with time due to various reasons. These include (a) product inhibition (b) fall in substrate concentration to the extent that % saturation of the enzyme with the substrate changes significantly, (c) the product concentration increases and the substrate becomes depleted and the reaction velocity in the reverse direction may become significant, and (d) the operational stability of the enzyme may become a factor and enzyme may start getting inactivated. The presence of known or unknown reactive compounds present in the industrial grade substrates may contribute to this factor.

Hence, if the enzyme is being used for a bioconversion or biotransformation for an industrial application, knowledge of just initial rates is not sufficient. In fact, it can be misleading. So, it is very necessary that complete progress curve of the reaction is drawn under intended process conditions. This can be done at the laboratory scale. Even this picture may change when the process is scaled up to the pilot plant or industrial level. But that is a different issue.

Temperature optimum, pH optimum and thermal stability data

It is the characteristic of enzymes as biocatalysts that they perform best at a particular temperature and pH and thermal inactivation begins in a significant way beyond a certain temperature. Hence, information about these three characteristics is routinely expected in any research article describing a new enzyme. These issues are equally important in industrial enzymology as well. All three are discussed in most textbooks of biochemistry. However, each one requires a more careful consideration than frequently given.

Temperature optimum

The activity vs. reaction temperature typically forms a bell shaped curve. Initial increase is due to increase in reaction rates with increase in temperature. Beyond the optimum value, the activity declines as protein chain unfolds, the thermal inactivation sets in (Gupta, 1993). However, it is important to distinguish between two very different patterns of behavior. In some cases the dominant process is an equilibrium between native and denatured structures, so that catalytic rates decline straightforwardly beyond the optimum. However, in many cases irreversible inactivation processes (which may involve a reversibly unfolded form as an intermediate) occur on a timescale comparable with that of the assay. Under these circumstances the reaction progress at higher temperatures is strongly curved, as enzyme is inactivated. Then it is difficult to estimate a meaningful initial rate. Some studies will define activity based on a single time point measurement of product formed (or substrate consumed). In studies of temperature effects this is a particularly dangerous design. With progress curve in reality strongly curved, the estimate of "activity" (based on an assumption of linear progress) will be higher the shorter the choice of reaction time. As temperature increases, the rate at the shortest times may continue to increase due to normal thermal effects, but faster inactivation will increase curvature of progress. Hence the apparent "optimum temperature" will depend on the arbitrary choice of assay duration, being highest for the shortest assays.

- It is necessary that the buffer in which the thermal exposure is carried out is described completely. Ionic strength may play a role (see also Bisswanger, 2014). Presence of additives can significantly affect the temperature optimum. This includes presence of simple ions. Calcium ion, for example, affects both the activity and/or stability of several enzymes.
- The start of the reaction is taken from the moment assay mixture is placed at the particular temperature. The system may take some time in acquiring the temperature of the bath. Ideally, the buffer may be pre-incubated at the desired temperature and a small aliquot of the enzyme solution may be added to start the reaction.
- Protein aggregation is one possible mechanism of thermal inactivation and becomes significant at higher protein concentration. Some enzymes are more aggregation-prone than others. Similarly, many enzymes get inactivated in extremely dilute solution (non-specific adsorption to vessels is one possible factor). Hence, protein concentration at which the measurements are made should be explicitly stated. It may be desirable to fix a range of protein concentration which may be followed by all.

Thermal stability

Thermal stability is the most frequently studied parameter in order to assess the stability of the enzyme in general terms. It is not an incorrect trend in as much as a more thermostable enzyme is more likely to be stable under other harsh conditions as well, for example, when exposed to organic solvents. The inactivation mechanisms of an enzyme under all conditions involve presumably unfolding of the protein chain as the first common step (Gupta, 1993). However, in recent years, "native-like structures" are known to aggregate (Bemporad et al., 2012). At the same time, aggregation need not result in inactivation. As already mentioned, we have recently reported an aggregated form of α-chymotrypsin which shows higher activity in both aqueous buffers and non-aqueous media (Rather et al., 2012). Stabilization under extreme pH conditions is also a desirable goal in several cases. Stability of proteases under alkaline conditions, for example, is useful for incorporating these enzymes in detergents. Often, such stability or stabilization is reported when the biocatalyst prepared is dissolved or suspended in aqueous buffers. In terms of validity of the data, that is not a problem provided all conditions are properly defined. This is necessary since for a protein solution, stability strongly depends upon the concentration, the nature of the buffer and the presence of any other additive. From practical point of view, such data merely provides a rough guideline. In practice, one is more concerned with operational stability that is the stability of the enzymes in the presence of substrates, co-enzymes (if any) and products formed during the reaction. Often industrial-grade substrates are dirty, colored and suspensions. The impurities present in such substrate preparations can impact operational stability to a great extent.

A rather common problem in reporting of stability studies is that the central principle of the experimental design is not made clear. One possible design is to pre-incubate the enzyme for a defined period under the challenging conditions (e.g. high temperature), then add substrates under those same conditions so as to determine the remaining activity. More commonly, following pre-incubation a portion of the enzyme will be assayed at some standard conditions, following cooling, dilution or similar. This design tests for irreversible changes that have occurred during pre-incubation. There is a case to be made for either design, but authors need to be clear which was followed. Of course, as noted, the best design may be to monitor the operational stability as the enzyme continuously converts substrates, but the more difficult experimental arrangements needed make this the least common choice.

As far as thermal stability data is concerned, there is an increasing trend to just give half-life data. This is an outcome of the necessity to keep the production cost of a research article low by reducing the length. Strictly speaking, the half-life data is valid only if the thermo-inactivation kinetics follows first order. More often than not, enzyme thermal inactivation kinetics is at least biphasic. In all such cases, reporting half-lives calculated from first-order kinetics should be avoided. Unfortunately, the poor peer review system has many times led to reviewers insisting that half-lives be calculated! Many decades back, the seminal work of Sadana's group had described thermal inactivation models to deal with all possible kinds of thermal inactivation kinetics (Sadana, 1991,1993).

Stability of enzymes in organic solvents

This is one area wherein one sees a complete confusion between storage stability and operational stability. In order to fully appreciate the extent of this, let us briefly examine the consequences of the presence of organic solvent on enzymes activity.

We should not overlook an old review by Singer which provides information about solubility of proteins or enzymes in organic solvents (Singer, 1963). Given the current knowledge about influence of a_w or $[H_2O]$ in the reaction media during enzymatic catalysis (Halling, 1992, 1994; Valivety et al., 1992), it may be useful to run a control on the % of the dissolved enzyme under exact solvent conditions. This should provide the information about the contribution of soluble enzyme component towards overall catalysis.

When 0–10% water miscible organic solvent is present in the aqueous media, considerable increases in reaction rates have been reported (Batra and Gupta, 1994). The phenomenon is far from clearly understood but a more flexible enzyme structure, change in the medium dielectric constant etc. have been implicated. It is not uncommon to use this small concentration of water-miscible organic solvent to facilitate solubilization of organic substrates. Wherever necessary, a control examining effects of the organic solvent (at that concentration) on enzyme activity can be run with a more water soluble substrate.

Enzymes undergo denaturation when the organic solvent (water miscible) concentration is in the range of 10–90% (these ranges are approximate numbers, the actual value varies from enzyme to enzyme). Some organic solvents are more damaging than others. Parameters like denaturation capacity have been defined and examined (Khmelnitsky et al., 1991).

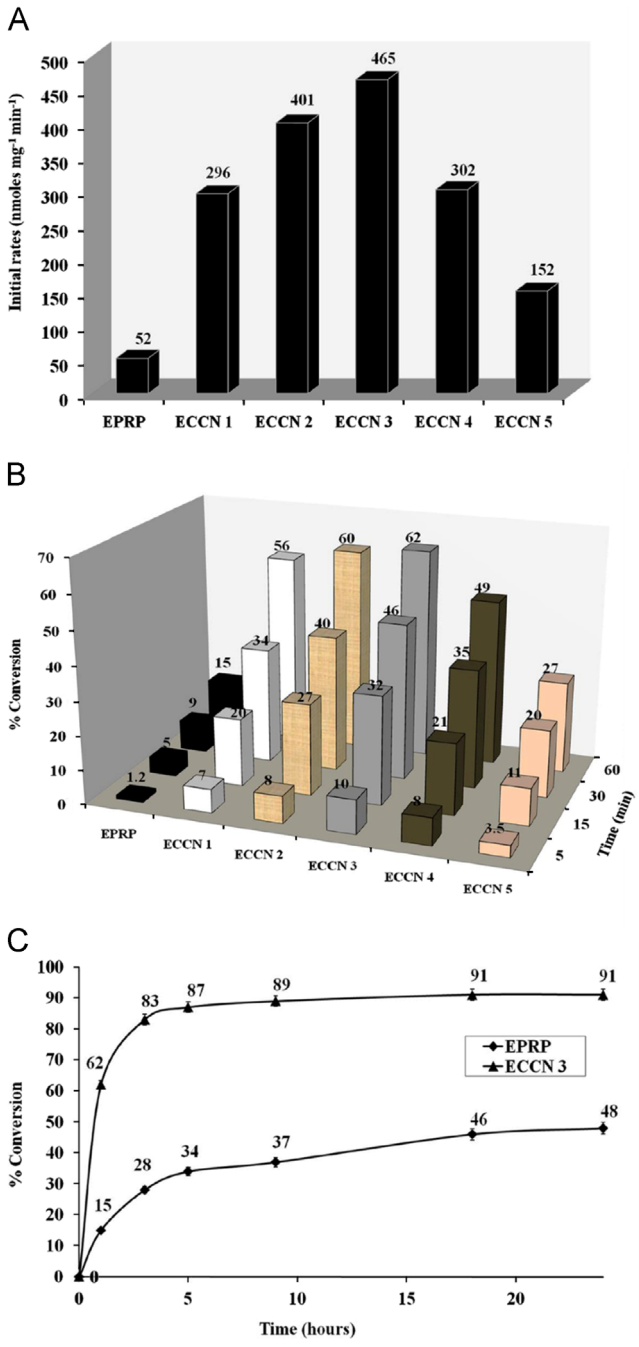

Figure 2 (A) Initial rates (B) % conversions (C) % conversion over 24 h for the transesterification reaction. Precipitation of α-chymotrypsin over a stirred suspension of Fe_3O_4 nanoparticles led to the formation of enzyme coated clusters of nanoparticles (ECCNs). The free enzyme precipitates were called enzyme precipitated and rinsed with propanol (EPRP). These formulations were used to carry out the reaction. The reaction was carried out between *N*-acetyl-L-phenylalanine ethyl ester and *n*-propanol in anhydrous *n*-octane as a solvent with different enzyme formulations. The initial rates are in terms of mg protein precipitated in each case. The experiments were carried out in duplicates and the error between each set of readings was with 3%.
(This was reproduced with permission from *Chemistry Central Journal*, Mukherjee and Gupta, 2012, 6(133), 1–12.)

Water immiscible organic solvents form a different phase in this range of concentration and two-phase systems are used for carrying out bioconversions or biotransformations (Mattiasson and Holst, 1991). The advantage offered is that product inhibition can be relieved by product moving to a phase different from where the catalysis is taking place. Furthermore, there may be desirable shifts in the equilibrium position in the non-aqueous phase, for example esterification by reverse hydrolysis can become favorable. It also offers the possibility of working with high concentration of water insoluble substrates by dissolving the substrate in the organic solvent rich phase. In such a situation, the reaction starts with the amount of the substrate which partitions to the aqueous phase wherein the enzyme is placed.

Low water containing organic solvents as reaction media are claimed to offer number of advantages (Klibanov, 2001). Not all of these necessarily work with most systems. In these media, the low water activity adds a further contribution that shifts the equilibrium of reactions catalyzed by hydrolases in favor of synthesis (Clapes et al., 1990; Reslow et al., 1988). Unfortunately, after the initial excitement, it was soon realized that commercial preparations and lyophilized powders show very low catalytic activity. As high as 20% (w/w with respect to substrate) of the enzyme preparation has been routinely used. In the last two decades, some understanding of the structural aspects of enzymes function in low water medium has emerged (Carpenter et al., 1993; Gupta, 1992; Lee and Dordick, 2002; Roy et al., 2004). Efforts to design formulations which showed much higher activity than lyophilized powders have been described (Hudson et al., 2005; Kreiner et al., 2001; Lee and Dordick, 2002; Mukherjee and Gupta, 2012; Shah et al., 2006; Sheldon et al., 2005; Roy and Gupta, 2004) (Figure 2).

It is this issue which needs to be discussed at some length. Many biocatalyst preparations are described claiming that high initial rates and conversions displayed by these show higher stability of the enzyme preparation in the organic solvent media. At most, one can claim that operational stability may have contributed to the high value of conversions. In large number of cases, such preparations involve immobilization (Minteer, 2011; Torres-Salas et al., 2011) or dispersal of the enzyme over a larger surface (Karajanagi et al., 2004). In all likelihood, the reason behind the higher activity observed is reduction in mass-transfer constraints!

Similarly, while discussing low initial rates observed in a particular solvent, the conclusion that the enzyme is not stable in that particular solvent is not necessarily correct. It may be just that the enzyme has low activity in that solvent.

Moonlighting and promiscuity

The concept of defining the unit of an enzyme activity relies upon the assumption of biological specificity of enzymes. A protease will hydrolyze a peptide bond and a substrate like casein can be used for measuring its activity. This system has worked reasonably well over the years.

The first sign of the problem arose when enzymes were used in non-aqueous media. In such media, proteases may catalyze the formation of peptide bonds. Even their specificity is not same as in aqueous media (Gupta, 1992). Suppose, an author reports that upon immobilization on a particular matrix, it is possible to have a highly active enzyme in low-water media. The literature has very large number of such reports in even many impressive journals and this number continues to grow at a very large rate. It is quite common to offer a comparison of activity with that displayed by a lyophilized powder of the same enzyme. However, the large enhancements reported here mainly reflect the very poor activity of simple lyophilized powders, as discussed earlier. In non-aqueous media, the comparison of the activity of immobilized preparations with the free enzyme is generally not meaningful (unlike in aqueous media where it is standard practice). A comparison of specific activity in the same medium with previously reported effective preparations would be useful, but is rarely presented. A comparison with activity in aqueous media can be informative, but it must be acknowledged here that this is often not as straightforward as would be hoped – for example, a hydrolytic reaction used in an aqueous assay may hardly proceed in non-aqueous conditions.

The second important complicating issue is that right now many substrates are being used to report efficiency of the biocatalyst for a particular type of reaction in low water media. So, different reports on a trans-esterification between an ester and an alcohol may use different esters and/or different alcohols. As such reactions strongly depend upon the reaction medium, even same reaction with identical substrates cannot be compared if different solvents were used.

According to Hult and Berglund (2007) as enzymes show different specificity in such unconventional media, such behavior can be called a case of condition promiscuity.

A more troublesome situation is vis-à-vis catalytic promiscuity (Khersonsky and Tawfik, 2010). According to the EC system of nomenclature (Nomenclature Committee of IUBMB, 1992M; see also http://www.chem.qmul.ac.uk/iubmb/enzyme/), enzymes are classified into six main classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Hence, lipases are hydrolases. Aldol condensation, on the other hand, is carried out by lyases, aldehyde-lyases has been assigned the number 4.1.2 (Nomenclature Committee of IUBMB, 1992). However, lipases have now been shown to catalyze not only aldol condensation, but also the Mannich reaction, Michael addition, Morita-Baylis-Hillman reaction as well (Hult and Berglund, 2007; Kapoor and Gupta, 2012; Lai et al., 2010; Li et al., 2008)! So, to start with we have a problem with the classification. Khersonsky and Tawfik (2010) have made some suggestions in the regard. In many cases, these promiscuous reactions involve high catalytic efficiency which is in the same range as seen in normal enzyme catalyzed reactions. Babtie et al. (2010) have discussed this and point out that rate accelerations $(k_{cat}/K_m)/k_2$ of up to 10^{18}-fold are known. In many other cases, protein engineering and directed evolution has been successfully used to induce catalytic promiscuity (Khersonsky and Tawfik, 2010). Many of these reactions are industrially important. Large number of reports regarding catalytic promiscuity deal with reactions carried out with industrial preparations of lipases (Busto et al., 2010; Kapoor and Gupta, 2012).

While catalytic promiscuity involves the active site of the enzyme, moonlighting by proteins can involve different parts of the enzyme molecule (Jeffery, 1999). The phenomenon of moonlighting constitutes a definite shift from the well-known one gene-one protein-one function paradigm. The different functions of a moonlighting protein can be displayed: in two different locations in the cell (one can be even intracellular and another extracellular); by a change from the monomer to oligomer structure, in different cell types or even with a change in ligand or substrate concentrations (Jeffery, 2009). While few examples of moonlighting involve different catalytic activities, in larger number of cases the different activities encompass non-catalytic functions like repressor, growth factor, receptor, inhibitor, chaperone and regulator activities (Jeffery, 1999, 2009).

Apparently new enzymes continue to evolve. Atrazine chlorohydrolase (which degrades herbicide atrazine) has evolved (from melamine hydrolase) between 1950 and 1990 (Janssen et al., 2005). Directed evolution, of course, is being extensively used to obtain enzymes which tailored specificity (Arnold and Georgiou, 2003a,b).

All the different phenomena and observations discussed in this section have a common message: old classification and old way of reporting data on enzyme catalyzed reactions may not be adequate. In some cases, a little tweaking of guidelines may work. Eventually, we would need to evolve new guidelines (see also Tipton et al., 2014).

Conflict of interest statement

None of the authors have any conflict of interest.

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